**In vivo** inhibition of tumor progression by 5 hydroxy-1,4-naphthoquinone (juglone) and 2-(4-hydroxyanilino)-1,4-naphthoquinone (Q7) in combination with ascorbate

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**A B S T R A C T**

The purpose of the study was to obtain further in vivo data of antitumor effects and mechanisms triggered by juglone and Q7 in combination with ascorbate. The study was done using Ehrlich ascites tumor-bearing mice. Treatments were intraperitoneal every 24 h for 9 days. Control group was treated with excipient. Previous tests selected the doses of juglone and Q7 plus ascorbate (1 and 100 mg/kg, respectively). Samples of ascitic fluid were collected to evaluate carbonyl proteins, GSH and activity of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase. Hypoxia inducible factor HIF-1α, GLUT1, proteins driving cell cycle (p53, p16 and cyclin A) and apoptosis (poly-ADP-polymerase PARP, Bax and Bcl-xL) were assessed by western blot. Tumor cells were categorized by the phase of cell cycle using flow cytometry and type of cell death using acridine orange/ethidium bromide. A glucose uptake assessment was performed by liquid scintillation using Ehrlich tumor cells cultured with 14C-deoxyglucose. Treatments caused increased protein carbonylation and activity of antioxidant enzymes and decreased levels of GSH, HIF-1α, GLUT1 and glucose uptake in tumor cells. They also caused increased number of tumor cells in G1, p53 and p16 activation and decreased cyclin A, but only when combined with ascorbate. Apoptosis was induced mostly when treatments were done with ascorbate, causing PARP and Bax cleavage, and increased Bax/Bcl-xL ratio. Juglone and Q7 in combination with ascorbate caused inhibition of tumor progression in vivo by triggering apoptosis and cell cycle arrest associated with oxidative stress, suppression of HIF-1 and uncoupling of glycolytic metabolism.

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**1. Introduction**

Some important drugs used for the treatment of cancer belong to the quinone class of organic compounds (e.g. daunorubicin and doxorubicin) [1]. Several quinoid compounds keep on being isolated or synthesized and screened for antitumor activities. The studies have also evaluated the interactions occurring between some quinoid compounds and ascorbate [2,3]. In fact, it has been shown that ascorbate at pharmacological concentrations enhances the activity of drugs such as doxorubicin, among others [4].

Some 1,4-naphthoquinones seem to be promising for targeting cancer cells [5]. In general, these quinones possess a redox potential that allows inducing an oxidative stress responsible for damage in tumor cells [6]. As part of our ongoing studies, a series of 1,4-naphthoquinones, including juglone (5-hydroxy-1,4-naphthoquinone), Q7 (2-(4-hydroxyanilino)-1,4-naphthoquinone),...
Q9 (2-(4-methoxyanilino)-1,4-naphthoquinone) as well as other 3-acetyl-2-phenylamino-1,4-naphthoquinones have been synthesized and screened for antitumor activities with or without ascorbate. Initially, a series of in vitro assays were performed. These investigations have shown inhibition of cell proliferation and migration by oxidative stress from ascorbate-driven juglone redox cycling in human bladder-derived T24 cells in culture [7]. In addition, it was previously shown that the antiproliferative effects of Q7 and Q9 are potentiated by ascorbate and associated with the appearance of a senescent phenotype in T24 cells [8]. The formulations prepared with juglone, Q7 and Q9 in combination with ascorbate were tested later in vivo using Ehrlich ascites tumor-bearing mice. These screening assays showed that the potentiating effect of ascorbate was reproduced in vivo in the cases of juglone and Q7. Q7 plus ascorbate caused up to 60% of inhibition of tumor and the largest extension of survival of mice. Therefore, taking into consideration some previous studies from our laboratory, a series of 1,4-naphthoquinones were left behind, whereas juglone and Q7 plus ascorbate were kept in the study because they caused the most promising antitumor activity, causing DNA damage and inhibition on pAkt in Ehrlich ascites tumor in mice [9].

These previous data provided the basis for maintaining the investments on further studies in order to understand better the effects caused in vivo by juglone and Q7 in combination with ascorbate. In the current phase, our approach took into account some potential actions on an important part of the antioxidant system, as well as the effects on the glycolytic metabolism and tumor survival under hypoxia by assessing HIF-1α, glucose transporters GLUT1 and glucose uptake in tumor tissue of Ehrlich tumor-bearing mice. Therefore, the aim of this work was to obtain further pharmacological data of the antitumor effects and mechanisms triggered by juglone and Q7 with and without ascorbate in vivo. This manuscript reports data showing that these treatments induced oxidative stress associated with cell cycle arrest and tumor cell death in vivo triggering changes in terms of proteins involved in cell cycle control and also in apoptosis.

2. Methods

2.1. Drugs

Juglone 97% (Cat. H47003) and sodium ascorbate ≥98% (Cat. A7631) were purchased from Sigma-Aldrich. Q7 was synthesized by amination of 1,4-naphthoquinone with 4-aminophenol, under aerobic conditions using CeCl3·7H2O as the Lewis acid catalyst, as previously described by Benites et al. [10].

2.2. Animals

Male BALB/c inbred mice (20–22 g) were housed under controlled conditions, receiving water and food ad libitum. This research was conducted in accordance with internationally accepted principles for laboratory animal use and care (NIH publication # 85–23, revised in 1985). This experimental protocol was approved by the local ethics committee of Universidade Federal de Santa Catarina, Brazil (CEUA-PP00784).

2.3. Tumor induction and treatment

On day zero, Ehrlich carcinoma cells (5 × 10⁶) were inoculated into the abdomen of mice (n = 12). Twenty-four hours later, the treatments got started. The treatments were done via intraperitoneal injections every 24 h for 9 days. The control group was treated only with excipient (saline - DMSO 0.1%). Previous tests were conducted to select the doses of juglone and Q7 plus ascorbate (1 and 100 mg/kg, respectively). Ascorbate was administered at doses 100-fold higher than the doses of juglone or Q7 [9]. Two hours after the last dose, samples of ascitic fluid were collected for analysis.

2.4. Biomarkers of oxidative stress in Ehrlich tumor tissue

Samples of ascitic fluid were centrifuged (5000 g for 5 min). Supernatants were diluted (1:5 v/v) in buffer containing 100 mM sodium phosphate, 150 mM NaCl and 0.1% Triton X-100 (pH 7.4) and acidified in trichloroacetic acid (TCA 12%, 1:5 v/v) for the measurement of reduced glutathione (GSH). Oxidative damage to proteins was quantified as carbonyl proteins as described by Levine et al. [11]. Carbonyl groups of proteins react covalently with 2,4-dinitrophenylhydrazine, which triggers the formation of a 2,4-dinitrophenylhydrazone product. The reaction was followed by spectrophotometric quantification of the acid hydrazones at 370 nm. The content of GSH was estimated as described by Beutler et al. [12] using the reaction of small thiols in the sample with dithiobisnitrobenzoic acid. The resulting yellow thiolate formation, proportional to the content of GSH, can be quantified spectrophotometrically at 412 nm. Catalase activity was determined through the method described by Aebi [13] based on the decomposition of H₂O₂ and the decrease in absorbance at 240 nm. Superoxide dismutase (SOD) activity was measured monitoring the oxidation of adrenaline to adrenochrome as described by Misra and Fridovich [14]. Glutathione peroxidase (GPx) activity was measured using the method described by Flohé and Günzler [15]. Tert-butylic hydroperoxide was used as substrate to be reduced by GPx in a reaction that uses GSH as a reducing agent forming GSSG. By using glutathione reductase (GR), GSH can be regenerated from GSSG in a reaction that uses NADPH as a reducing agent. The rate of NADPH oxidation is proportional to the activity of GPx that was quantified at 340 nm. This principle was followed as well to estimate GR activity using the method described by Calberg and Mannervick [16]. All results were normalized to the protein content using the method of Lowry et al. [17].

2.5. Immunoblotting assays

Samples of ascitic fluid (250 μL) were centrifuged (5,000 g for 5 min). The supernatants were discarded and the pellet washed in phosphate buffered-saline (PBS) and centrifuged again. Whole cell lysates were prepared in RIPA lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM phenylmethylsulfonyl fluoride) supplemented with 1% protease inhibitor and 3% phosphatase inhibitor cocktails. After further denaturation in Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), equal amounts of protein (30 μg) were subjected to SDS-PAGE, followed by electroblotting to PVDF membranes. After blocking and washing, the membranes were incubated overnight with the primary antibodies, washed again and further incubated with the secondary antibodies (1 h). Immunodetection was performed using the enhanced chemiluminescence (ECL) detection kit (Millipore, USA) for HRP-coupled secondary antibodies. Beta-actin served as a loading control. The primary antibodies were: Rabbit polyclonal antibodies from Santa Cruz® raised against HIF1α (Cat. sc-10790), GLUT1 (Cat. sc-7903), cyclophilin A (Cat. sc-596), PARP (Cat. sc-7150), p53 (Cat. sc-6243), actin (Cat. sc-7210). Mouse monoclonal antibodies for detection of protein Bax (Cat. sc-7480) and Bcl-2 (Cat. sc-8392), Rabbit polyclonal antibody for detection of p16 (Cat. 4824) from Cell Signaling®. The secondary antibodies: Polyclonal goat anti-rabbit IgG antibody (Cat. AP132P) and polyclonal goat anti-mouse IgG antibody (Cat. AP181P), both peroxidase conjugated from Merck Millipore. Western blots were quantified by densitometry.
Fig. 1. Biomarkers of oxidative stress measured in tumor tissue from Ehrlich ascites tumor-bearing mice treated with juglone or Q7 with or without ascorbate and the saline-treated control. Protein carbonylation (A); reduced glutathione (GSH) concentration (B) and activity of antioxidant enzymes: catalase (CAT) (C); superoxide dismutase (SOD) (D); glutathione peroxidase (GPx) (E) and glutathione reductase (GR) (F). (*), (**) and (***) denote difference at \( p < 0.05 \), \( p < 0.01 \) and \( p < 0.001 \) compared to the control or indicated treatments.

Fig. 2. Data of immunoelectrophoresis against the hypoxia inducible factor HIF-1α and glucose transporters GLUT-1 in samples of tumor tissue from Ehrlich ascites tumor-bearing mice of the control and mice treated with juglone or Q7 with or without ascorbate (A). Data of \(^{14}\)C-deoxyglucose uptake assay performed with ex vivo Ehrlich tumor cells treated in culture with juglone or Q7 with or without ascorbate. The control was cells treated only with fresh medium (B). (*), (**) and (***) denote difference at \( p < 0.05 \), \( p < 0.01 \) and \( p < 0.001 \) compared to the control or indicated treatments.
using the freeware Image J by Wayne Rasband from National Institute of Health (USA).

2.6. Cell cycle arrest evaluated by flow cytometry

Tumor cells were assessed according to the DNA content measured by flow cytometry using a fluorescent dye that binds DNA, a PI/RNAse solution kit from Immunostep®. Ehrlich carcinoma cells \((5 \times 10^5)\) were carefully washed with PBS, pelleted and fixed by rapid submersion into ice-cold ethanol (70%) with vortexing. After overnight fixation at \(-20^\circ C\), cells were washed with PBS, pelleted, suspended and incubated with PI/RNAse solution. Finally, cells were evaluated by the FACS Canto II (BD Biosciences) cytometer. Data were processed using the Flowing Software 2.5.

2.7. Acridine orange/ethidium bromide staining

Samples of ascitic fluid (25 μL) were stained with a solution (5 μL) of acridine orange (200 μM) and ethidium bromide (250 μM). Afterwards, cells (300) were categorized through microscopy. According to this method, viable cells appear uniformly green. Early apoptotic cells stain green and contain bright green spots on the nuclei. Late apoptotic cells also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, these cells show condensed and often fragmented nuclei [18].

2.8. Ex vivo glucose uptake assay

Ehrlich ascites tumor cells from a non-treated mouse were incubated in Dulbecco modified Eagle’s medium (Gibco® Cat. 11965–092) supplemented with 10% fetal bovine serum in a CO₂ incubator (5% CO₂, 95% air humidity at 37 °C). Later, the cells were divided in cell culture flasks containing \(8 \times 10^5\) cells/each and fresh medium for the control or medium containing juglone or Q7 (10 μM) with or without ascorbate (1 mM) plus \(^{14}\)C-deoxyglucose (0.1 μCi/mL) and incubated for 1 h. Then, cells were centrifuged (1000 g 8 min) and double-washed with PBS, when NaOH 0.5 M (200 μL) were added to each tube. Radioactivity was measured by liquid scintillation using a LKB rack beta liquid scintillation spectrometer (model LS 6500, Beckman Coulter®). The protein concentrations were determined by the Lowry method and the results expressed as nmol glucose units/mg of protein [19].

2.9. Data analysis

Data were shown as means ± standard deviation and analyzed

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**Fig. 3.** Evaluation on the cell cycle in remaining tumor cells collected from Ehrlich ascites tumor-bearing mice treated with juglone or Q7 with or without ascorbate and the saline-treated control. Percentage of tumor cells in Sub G1, G1, S and G2/M (A) and (B). Data of immunoelectrophoresis against proteins involved in cell cycle control, namely p53, p16 and cyclin A (C). (**) and (***) denote difference at \(p < 0.01\) and \(p < 0.001\) compared to the control.
by the analysis of variance test followed by the Bonferroni test. Comparisons and graphs were done using the GraphPad Prism software. Values of \( p < 0.05 \) were considered statistically significant.

3. Results and discussion

Ourique and collaborators [9] have previously reported binding and oxidative damage on DNA as well as increased lipid peroxidation in Ehrlich ascites tumor cells from mice treated with juglone and Q7 plus ascorbate. The interactions with DNA were considered important because DNA is the target for most anticancer drugs [20]. Lipid peroxidation is a marker of oxidative stress that relies on the evaluation of damage induced on membrane lipids [21]. In order to understand better the causes and consequences of oxidative stress induced by juglone and Q7 plus ascorbate in different tumor cell compartments, it was also important to evaluate other markers, such as modification on proteins and the influence on the activity of antioxidant enzymes. Juglone and Q7 induced oxidative damage on tumor cell proteins detected as increased levels of protein carbonylation, while such damage was even higher when juglone and Q7 were administered together with ascorbate (Fig. 1A).

Existing evidences indicated that, depending on the case, the administration of a quinoid compound in combination with ascorbate may trigger more than one type of cellular response. However, it seems consensus that the main mechanistic feature must have to do with the induction of oxidative stress in which the cancer cells usually deficient in antioxidant defenses are preferably activated to die [22,23]. Ascorbate can drive redox cycle of some quinoid compounds. For example, it was demonstrated that a redox cycling occurring between ascorbate and menadione (another 1,4-naphthoquinone) cause over generation of hydrogen peroxide, which seems to be the major reactive species resulting from this combination [24]. Hydrogen peroxide may also be within the major reactive species resulting from juglone and Q7 plus ascorbate administered in vivo because markers related to the antioxidant defenses whose primary role is hydrogen peroxide scavenging were altered in tumor tissue of Ehrlich ascites tumor-bearing mice. These findings favors the idea that juglone and Q7 can induce a widespread oxidative stress in tumor and their combination with ascorbate resulted in enhanced oxidative damage associated with increased activity of antioxidant enzymes, such as SOD activity. SOD dismutates \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \), in this way continuously generates \( \text{H}_2\text{O}_2 \) that must be adequately metabolized by CAT and GPx. To date three unique and highly compartmentalized mammalian superoxide dismutases have been characterized. SOD1 is found almost exclusively in cytoplasmic spaces. SOD2 is initially synthesized containing a leader peptide, which targets a manganese-containing enzyme in the mitochondrial spaces. SOD3 is synthesized containing a signal peptide that directs this enzyme to extracellular spaces [25]. The activity of total SOD is shown in Fig. 1D. The treatments also caused GSH consumption (Fig. 1B) and induction of CAT, GPx and GR (Fig. 1C, E, F), which are all involved in \( \text{H}_2\text{O}_2 \) scavenging [26]. GSH is found in extracellular spaces and intracellular GSH, GPx and GR are mainly in the cytosol and mitochondrial compartments [27,28]. In animals, catalase is an intracellular enzyme located mainly in peroxisomes [29].

Uncontrolled cell proliferation in tumors leads to colonizing areas at increasing distance from blood vessels, which causes hypoxia. As hypoxia arises, the activation of transcription factors, like HIF-1 occurs through dimerization. HIF-1β is expressed constitutively, whereas the expression of HIF-1α is \( \text{O}_2 \)-dependent and regulated through several key ways [30]. During hypoxia, HIF-1 dimerizes and its activation regulates gene expression particularly relevant to cancer cells. HIF-1 activation causes the production of angiogenic factors, chemoresistance and high levels of some glycolytic proteins, such as glucose transporters (GLUTs) [31,32]. Therefore, several strategies for suppressing HIF-1 activity have

Fig. 4. Percentage of tumor cells categorized into viable, apoptotic or necrotic ones according to the acridine orange/ethidium bromide staining (A). Samples of tumor tissue were collected from Ehrlich ascites tumor-bearing mice treated with juglone or Q7 with or without ascorbate and the saline-treated control. Western blots corresponding to PARP integrity and proteins involved in apoptosis Bax and Bcl-xL in these samples (B). Ratio of Bax/Bcl-xL quantified by densitometry (C). (**) denotes difference at \( p < 0.001 \) compared to the control or indicated treatments.
been evaluated in cancer treatment [33].

The treatments with juglone, Q7 and ascorbate alone caused decreased levels of HIF-1α and GLUT1. However, considering GLUT1 inhibition, the potentiating effect of ascorbate was verified only in the combination with Q7 (Fig. 2A). In fact, ascorbate participates in the metabolic process driving HIF-1α degradation. Ascorbate is a co-factor of prolyl hydroxylases that use O2 to hydroxylate HIF-1α and prevent its dimerization with HIF-1β, and consequently, HIF-1 activation. This pathway ends up in HIF-1α ubiquitination and proteasomal degradation [34].

Considering data suggesting that the treatments caused decreased GLUT1 (Fig. 2A), therefore it was valid to verify whether the treatments would be able to inhibit the glycolytic metabolism and GLUT1. However, considering GLUT1 a co-factor of prolyl hydroxylases that use O2 to hydroxylate HIF-1α, and consequently, HIF-1 activation. This pathway ends up in HIF-1α ubiquitination and proteasomal degradation [34].

Another aspect involved in strategies of cancer therapy is related to the effects on key mediators of cell cycle and proliferation. Compounds capable to stop cell cycle and prevent tumor cell proliferation have been considered to supplement the conventional cytotoxic chemotherapy [35]. The treatments carried out with juglone or Q7 caused increased number of tumor cells in G1, but only when combined with ascorbate, as shown in Fig. 3A and Fig. 3B where sub-G1 area show dead cells classified in Fig. 4A. Accordingly, the expression of p53 and p16, both inhibitors of cell cycle progression, was also increased only when juglone and Q7 were administered together with ascorbate (Fig. 3C). The expression of p53 and p16 inhibits phosphorylation of pRb, thus preventing cells to advance to S phase [36]. Moreover, cancer cell cycle progression from G1 requires cyclin A [37], and we were able to demonstrate that again, only when in combination with ascorbate, juglone and Q7 decreased cyclin A significantly (Fig. 3C).

Previously, the mechanisms of cell death induced by these treatments were assessed in vitro. Ji et al. [38] have demonstrated that juglone induced apoptosis in human gastric cancer SGC-7901 cells via the mitochondrial pathway. In the current study, juglone, Q7 and ascorbate alone caused apoptosis detected in Ehrlich tumor cells from mice (Fig. 4A). Once again, the number of cells in apoptosis was higher when mice were treated with the compounds in combination. Moreover, these results suggest apoptosis occurring through the detection of PARP cleavage and increased ratio of proteins Bax/Bcl-2 (Fig. 4B,C). It is known that PARP enzyme has some relationship in cell death mediated by p53. PARP has a DEVD sequence that can be recognized and cleaved by the caspases during apoptosis. The cleavage of PARP is a hallmark of apoptosis [39]. The protein Bax, which is essential for initiating the intrinsic apoptotic pathway, was cleaved in samples of Ehrlich tumor cells from mice (Fig. 4B). The proteolytic cleavage of Bax was reported to occur in tumor cells treated with various chemotherapeutic agents that activate the intrinsic apoptotic pathway [40,41] and some evidences indicate intracellular ROS accumulation to Bax activation and cleavage with subsequent apoptotic cell death [42].

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